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Effects on the Enzymes Production from Different Mixes of Agro-Food Wastes

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The solid state fermentation (SSF) is increasingly being used for the production of high value-added products using agro-food wastes as substrate. In fact, these residues which are available in large quantities and at low cost, have usable nutrients for the microorganism growth and seem to reproduce the natural habitat of filamentous fungi. In recent years, there have been significant additions to the science and engineering knowledge of SSF. Unlike systems SmF (Submerged Fermentation), the SSF are still able to achieve a high degree of development, mainly because of the problems associated with the beds solids, such as poor mixing of the materials and heat transfer.
For this reason, we have been further studied the efficiency of the fermentation process through mechanical treatments of extrusion of the substrate (SSF dynamics), able to promote the availability of substrate, the exchange of oxygen on the surface, the activity produced and the recovery enzyme. In the presence of this process, was observed an increase on the production of several enzyme activities. In the third week of fermentation of grape stalks, the caffeoyl and feruloyl esterase showed a considerable increase of almost 3 and 4 times (respectively), with respect to the values obtained previously in static SSF.
To increase the enzymatic activities produced by Pleurotus ostreatus in dynamic SSF (cellulase, xylanase, laccase, pectinase and arylesterase (caffeoyl esterase and feruloyl esterase)), we evaluated the effect of the mixes of agro-food wastes as substrates (grape stalks, grape seeds, and wheat bran) already previously used individually.
The different enzymatic production by the P. ostreatus probably depends on the different chemical-physical composition of the substrates. Furthermore was noted that in the mixes of vegetable matrix, the different substrates can act both as inducers that as inhibitors of certain classes enzymatic, in relation to the specificity of each individual matrix. One of the examples is the presence of grape seeds or stalks in the mix, which seem to counteract the inductive action of the wheat bran in the synthesis of cellulase and xylanase.

1. Introduction

Using of agro industrial residues as substrates in solid state fermentation (SSF) processes provides an alternative avenue and value-addition to these otherwise under- or not-utilized residues. The lignocellulose is the main structural constituent of plants and represents the primary source of renewable organic matter on earth. It can be found at the cellular wall, and is composed of cellulose, hemicellulose and lignin, plus organic acids, salts and minerals (Hamelinck et al., 2005).

The agro-food residues are frequently utilized in SSF because they are considered the best substrate for this process. In fact, these residues are available in large quantities at low cost, have usable nutrients for the microorganism growth and seem to reproduce the natural habitat of filamentous fungi, such as for example the Pleurotus ostreatus. The filamentous fungi are considered the better adapted organisms for SSF, since their hyphae can grow on the surface of particles and are also able to penetrate through the inter particle spaces, and then, to colonize it (Muller dos Santos et al., 2004).

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White-rot fungi such as Pleurotus ostreatus is able to produce a wide range of extracellular enzymes to degrade complex lignocellulosic substrates. Several agro industrial wastes are commonly used for this purpose, such as sugarcane bagasse, wheat bran, corn cob and straw, rice straw and husk, soy bran, barley and coffee husk (Sanchéz, 2009). For decades, such enzymes have been used in the textile, detergent, pulp and paper, food for animals and humans (Bocchini et al., 2003; Graminha et al., 2008).
The hydrolytic action of enzymes requires a substrate characterized by a good bioavailability biological attack. The structure of the plant cell wall polysaccharide is a network rather complex and dependent on their type (monocot or dicot). The ability of hydrolytic enzymes to degrade the plant cell walls depends on their ability to access the primary structure. For this reason the pre-mechanical and heat treatments are well known to promote the opening of the fibres of the matrix rather than to swell it favouring the water absorption. Another variable that is essential for SSF is the free water presented in the medium. Moisture content of substrate plays a vital role for the microbial growth and for effecting biochemical activities in SSF (Farias et al., 2014).
Stuart et al. (1999) observed improvements on the fungal growth of Aspergillus oryzae, which was attributed to the effects of transport phenomena as mixing, cutting, heat and mass transfer within the bed of the substrate, using a dynamic solid state fermenter with a rotating drum. The other side, an important aspect to be considered during the construction of a bioreactor is the sensitivity of the substrate and/ or the microorganism to shear forces generated by the mixing, because the enzymatic activity production could decrease or increase in the according to the fermented substrate used in a dynamic bioreactor.
The different chemical-physical composition of the substrates used in the SSF could influence on the type of enzymes produced by the fungus, also to induce a greater or lesser enzyme production. For this reason, is also interesting study the action of more than one vegetable matrix in the same fermentation, to observe if the enzyme production could be overproduced.

2. Materials and Methods

2.1 Solid state fermenter

Fifty grams of dry weight of substrate (wheat bran and grape seed) and 9.1g of dry weight of grape stalks (50 g wet weight) as such in a Pyrex bottle with a cotton cap were wetted with 50 mL of distilled water and sterilized by autoclaving. This substrate was inoculated using 9 g of Pleurotus ostreatus grow on malt extract agar. The fermentation takes place at 25 °C, in absence of light for a period of 21 days. About the sampling, this was done every 7 d opening the fermenter in the sterile hood and a specific quantity of water was added. The substrate was put into a screw extruder with cutting blade and extrusion holes of 5 mm diameter. The extract was recovered pressing and filtering the substrate. A new quantity of water was added to bring the humidity necessary for the subsequent fermentation. The extract (free-water) was centrifuged for 5 min at 13,000 rpm to remove the solid fraction and the enzymatic activities produced were determined.
The amount of water added to recovery the extract depends on the characteristics of the vegetable matrix and the mode in which the mycelium adapts to the growth substrate. The enzymatic activities of the substrates calculated per ml, were multiplied by the total volume of extract recovered to determine the total activity obtained from the fermentation process by eliminating in this way the effects of dilution due to the different conditions of recovery.

2.2 Determination of cellulase activities

The cellulase activities were determined by two enzyme assay:
Cellulase - cellulose functionalized:
The activity was determined reacting 1 mL of sample (extract), 2 mL of sodium citrate buffer 0.05 M at pH 4.8 and 50 mg of functionalized cellulose dyed with Remazol Brilliant Blue R. (RBBR), for 6 hours at 50°C. The cellulose functionalized made according the procedure described by Poincelot and Day (1972). The dye released from cellulose degradation by the enzyme action was detected by spectrophotometer at 595 nm against a control done in the same way as samples, but with distilled water instead of the extract.
Cellulase - reducing sugars:
To determine the reducing sugars released after the enzyme degradation of cellulose functionalized (described above), we was based on the procedure by Bailey et al. (1992). 0.4 mL of sample was added in 0.6 ml of 3,5-dinitrosalicylic acid (ADNS), boiled for 7 min in boiling water, the samples were cooled and centrifuged for 5 minutes at 13,000 rpm. The chromophore group created by the reaction between ADNS and reducing sugars, was detected by spectrophotometer at 550 nm against a control done in the same way as samples, but with 4 mL of distilled water and 0.6 mL of ADNS.

2.3 Determination of laccase activities

The laccase activity was determined following the method described by Setti et al. (1999), using an oxidative coupling reaction between 3-metil 2-benzotiazolinone idrazone MBTH and l'orto-methoxyphenol (guaiacol).
The reaction was prepared at 30 °C in a tube test with 3 mL of phosphate buffer (25 mM, pH 6.5) and 100 µL of extract. In this solution was added in rapid sequence and mixing with the vortex, 50 µL of guaiacol 500 mM in etanol and 500 µL of MBTH 0.05% (w/v) for an interval time necessary for the development of red coloured azo-dye compounds, which falls in the range of spectrophotometric reading, corresponding to the product of the reaction. The reaction was stopped adding in rapid sequence 500 µL of H_2SO_4 1 N and 1 mL of acetone. The samples were detected by spectrophotometer at 502 nm against a control tube done in the same way as samples, but adding distilled water instead of the extract. The absorbance is stable for up to 30 minutes.

2.4 Determination of xylanase activities

The xylanase activities was determined as reducing sugars following the method described by Bailey et al. (1992). In a test tube was added in 1.8 mL of xylan substrate 0.3 % prepared in citrate phosphate buffer at pH 5.0 and 0.2 mL of sample with enzymes produced (extract). The reaction was conduced at 30 °C mixing for 3 min. Subsequently, 0.4 mL of this solution was added in 0.6 mL of ADNS (3,5-dinitrosalicylic acid), boiled for 7 min in boiling water, cooled and centrifuged for 5 min at 13,000 rpm. The chromophore group created by the reaction between ADNS and reducing sugars, was detected by spectrophotometer at 550 nm against a control tube done in the same way as samples, but adding distilled water instead of the extract.

2.5 Determination of pectinase activities

The pectinase activities was determined as reducing sugars following the method described by Bailey et al. (1992). In a test tube was added in 1.8 mL of pectin substrate 0.3 % prepared in citrate phosphate buffer at pH 3.8 and 0.2 mL of sample with enzymes produced (extract). The reaction was conduced at 30 °C mixing for 3 min. Subsequently, 0.4 mL of this solution was added in 0.6 mL of ADNS (3,5-dinitrosalicylic acid), boiled for 7 min in boiling water, cooled and centrifuged for 5 min at 13,000 rpm. The chromophore group created by the reaction between ADNS and reducing sugars (galacturonic acid), was detected by spectrophotometer at 550 nm against a control tube done in the same way as samples, but adding distilled water instead of the extract.

2.6 Determination of arylesterase activities

To determine the caffeoyl esterase or feruloyl esterase activity, in a quartz cuvette with 0.9 mL of phosphate buffer 100 mM at pH 6.0, were added 0.1 mL of methyl ferulate or methyl caffeate 0.1 mM prepared in phosphate buffer and 0.05 mL of the extract. After adding the extract, the cuvette was mixed and immediately is reading a time drive at a spectrophotometer at 335 nm against a quartz cuvette with 0.9 mL of phosphate buffer. In which was detected the disappearance of methyl ferulate or methyl caffeate (substrate). The method was modified with respect to that reported by Giuliani et al. (2001).

3. Results and discussion

The physical characteristics of the substrate is directly related on the success of the solid state fermentation, because the particle size, shape, porosity and consistency for example, favour both gas and nutrient diffusion as well as the heat removal (Muller dos Santos et al., 2004) which is one of the major limiting factor for commercial viability of SSF since long.
To reduce the problems identified in the static fermentation, a dynamic solid state fermentation was developed, in which the inoculated substrate could be physically extruded either continuously or intermittently with a frequency from minutes to hours or days. In our experiments, the air was circulated through the headspace of the bed, but not blown forcefully through it. The fermentation was carried out in a substantially intermittent mixing bioreactor (dynamic fermentation) which operates like a tray bioreactor (static fermentation) during the static period and like a continuous rotating bioreactor during the phase of extrusion.
The results reported in this paper were obtained by fermentation with a duration of 21 d, making the extrusions weekly with subsequent recovery of the liquid fraction for the determination of enzyme activities produced.
Visual observation have evidenced a more uniform growth of the mycelium in the dynamic fermentation than in the static fermentation; although, a high damaging of the fungal mycelium would be expected in the intermittent bioreactor due to the strong stress on the cellular structure during the extrusion phase (Masutti et al., 2014).
According to the results obtained by Masutti et al. (2014), further tests were carried out in dynamic solid state fermentation. The hydrolytic and enzymatic activities were monitored to evaluate fungal growth and the enzymes production in different substrates in the presence of the mechanical treatment by a dynamic extrusion phase.

In Table 1, the enzyme activities obtained by dynamic solid state fermentation of the individual substrates (grapes stalks , grape seeds and wheat bran) are determined as total amount of activity extracted in 3 weeks of fermentation per 50 g (dry weight) of substrate. The values of the activities for the substrate grapes stalks have been reported to 50 g dry weight even if the fermentation is carried out with 9.1 g of dry weight (50 g wet), so as to be able to compare the yields of the different substrates.

*Table 1: Ligninolytic activities yields (µmol/min*g) of dry weight of substrates on the total recovered samples at weekly frequency until 3 weeks of fermentation*

	Yields of enzyme activities produced		
Enzymes	Wheat bran	Grape stalks	Grape seeds
Cellulase (reducing sugar)	5.5E+01	1.5E+00	3.1E+00
Xylanase	2.5E+01	2.0E+01	2.1E+00
Pectinase	2.1E+01	1.8E+01	2.4E+00
Laccase	1.6E-01	1.2E+00	1.7E+01
Caffeoyl esterase	1.8E+01	2.2E+02	7.7E+02
Feruloyl esterase	5.0E+01	4.0E+02	5.8E+02

The cellulase activities determined by cellulose functionalized resulted very low, so it is not reliable to make a comparison between different substrates and therefore were not reported in this paper.
The results highlighted a different capacity of the substrates to induce the production of different enzymatic activities and in particular, it is observed that the major activities are divided as follows: xylanase, pectinase and arylesterase from the fermentation of grape stalks, cellulase from wheat bran and laccase from grape seeds. It has been sensed then, an interesting simultaneous use of these substrates for possible complementation of enzyme production in a single fermentation. Interestingly, although the effect of a chemical species is well shown that it can induce the production of specific enzymes, the different specificities of complex natural matrices, such as those arising from food wastes, however, seem to maintain the same selectivity.
The above conclusions lead us to verify the possible synergistic effects of growth substrates as well as their characteristics of effector and/or inhibitors of enzymatic activity by Pleurotus ostreatus, were made on solid state fermentations with different mix of substrates.
The different mix of substrates were prepared using two matrices with 25 g of each one so as to keep the total 50 g of biomass used previously in all fermentations containing the individual substrates. The fermentation is carried out for 21 d and the measurements of enzyme activities were performed every seven days (weekly recovery of the liquid fraction).
Taking into account that the different mix of substrates were composed of 50 % by weight of each matrix, the enzymatic activities produced using the mix showed that the carbohydrases (such as cellulase and xilanase) were induced in the presence of wheat bran, which is a vegetable matrix of a typical monocot enriched in cellulose and hemicellulose (Figure 1). The pectinase activity was higher in the presence of the mix wheat bran-grape stalks, which were substrates that individually had expressed the most significant values for this class of enzymes. The inhibition effects were not observed using these substrates, as instead it was observed in others two mix in which the grape seeds were present. Biomass of grape seeds or grape stalks, when present in the mix, appeared to counteract the inductive action of the wheat bran in the synthesis of both cellulase and xylanase activities. This antagonistic action on carbohydrases seemed evident using grape seeds as also evidenced by the production of pectinase. The activity of laccase was mainly produced in the presence of the grape seeds being a matrix particularly enriched of lignin. The presence of grape seeds in the mix induced the production of laccase without undergoing a significant antagonistic effect of the other matrices.
The low of production of carbohydrase activities in the different mix could be explained with an inhibiting action of the grape seeds even if in the presence of wheat bran and grape stalks; however, the same grape seeds showed the induction of high enzymatic production of both caffeoyl esterase and feruloyl esterase. In particular, the feruloyl esterase activity presented an overproduction when using the mix wheat bran-grape seed, although individually the wheat bran had presented low values for this enzyme. In conclusion, on the one side the laccase activity seemed to depend on the presence of the lignin fractions, on the other side the carbohydrase activities seemed to be affected by the probably elevated levels of phenolic fractions in solution.
The arylesterase activities are typically induced by the presence of grape seeds, this effect seems reasonable since the arylesterase act to separate the fractions hemicellulose from lignin.

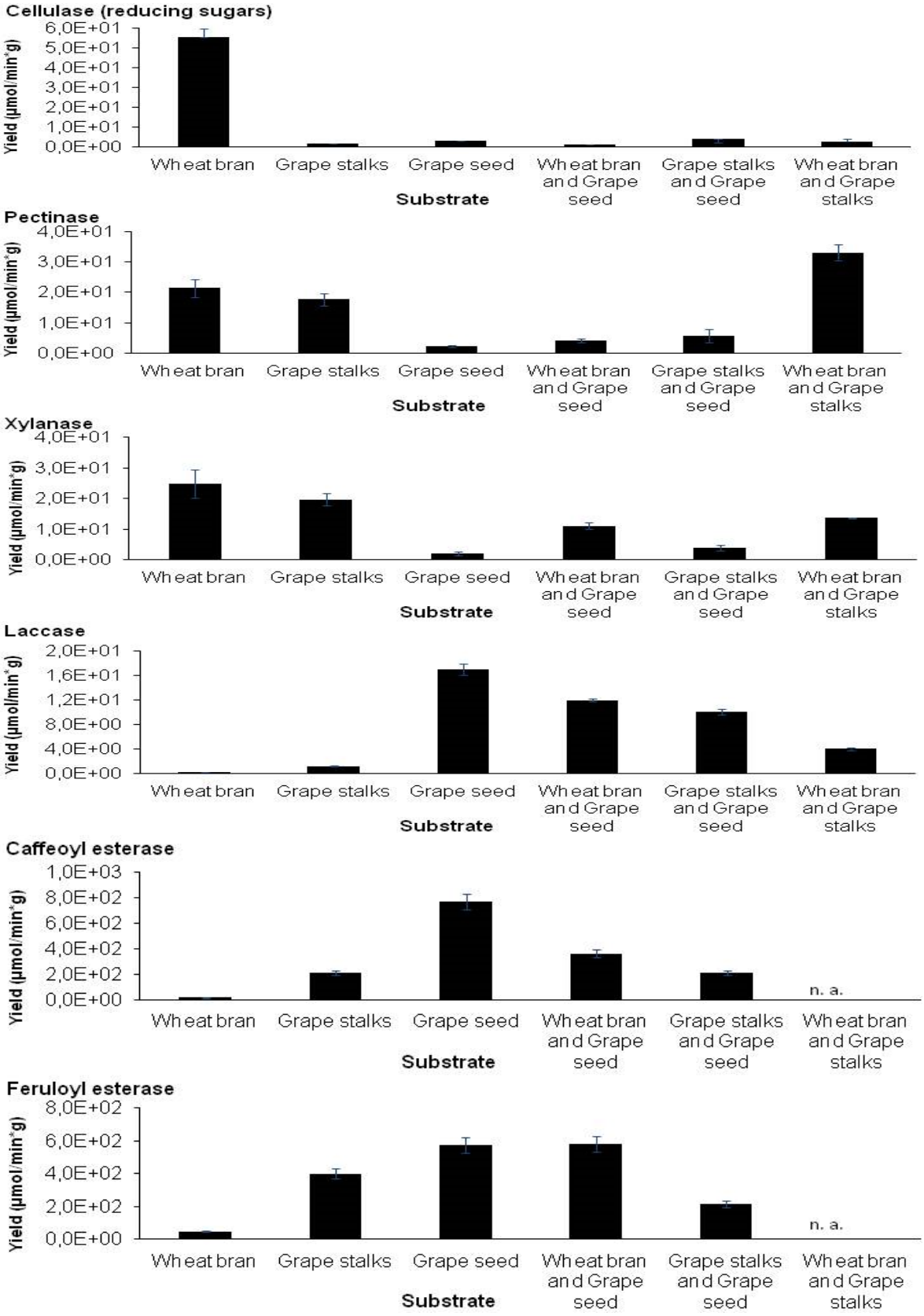

*Figure 1: Comparison of the yields of enzyme activities (mol/ min*g) obtained by the fermentation of individual substrates wheat bran, grape stalk and grape seed, and mix of substrates wheat bran-grape seed, grape stalks-grape seed, wheat bran-grape stalks, obtained in the first 3 weeks of fermentation. n.a.: not analysed.*

4. Conclusions

Our findings demonstrated that the enzymatic production of Pleurotus ostreatus in solid state fermentation depended to the presence of different vegetable matrixes as substrates. Fungus focuses its production according to the matrix on which was developed, inhibiting or inducing the release of enzymes needed for degradation of the substrate. The substrates performed their inducing and inhibiting actions even if they were into the mixes. In fact, is possible to talk about inhibitory effect on the carbohydrases in the presence of grape seeds and a strong induction of grape seeds on laccase and arylesterase. On the basis, adjusting the mix with a proper ratio of each type of substrate, it might be possible to overproduce different kind of enzymes.
This result can then be interpreted as an inhibitory action or modulating the enzymatic activity by matrices which play an antagonistic action.

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